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Fumagillin and Fumarranol Interact with *P. falciparum* Methionine Aminopeptidase 2 and Inhibit Malaria Parasite Growth In Vitro and In Vivo

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SUMMARY

The fumagillin family of natural products is known to inhibit angiogenesis through irreversible inhibition of human type 2 methionine aminopeptidase (MetAP2). Recently, fumagillin and TNP-470 were reported to possess antimalarial activity in vitro, and it was hypothesized that this inhibition was mediated by interaction with the putative malarial ortholog of human MetAP2. In this report, we have overexpressed and purified to near-homogeneity PfMetAP2 from bacteria, yeast, and insect cells. Although none of the recombinant forms of PfMetAP2 exhibited enzymatic activity in existing assays, PfMetAP2 proteins expressed in both yeast and insect cells were able to bind to fumagillin in a pull-down assay. The interaction between fumagillin and analogs with PfMetAP2 was further demonstrated using a newly established mammalian three-hybrid assay incorporating a conjugate between dexamethasone and fumagillin. Unlike human (Hs)MetAP2, it was found that PfMetAP2 is bound to fumagillin noncovalently. Importantly, a new analog of fumagillin, fumarranol, was demonstrated to interact with PfMetAP2 and inhibit the growth of both chloroquine-sensitive and drug-resistant *Plasmodium falciparum* strains in vitro. Antiparasite activity of fumagillin and fumarranol was also demonstrated in vivo using a mouse malaria model. These findings suggest that PfMetAP2 is a viable target, and fumarranol is a promising lead compound for the development of novel antimalarial agents.

INTRODUCTION

Cleavage of N-terminal methionine of newly synthesized proteins is an essential cotranslational modification process that is required for proper subcellular localization, folding, and activities for a large subset of cellular proteins (Boutin, 1997; Hu et al., 2007; Perrier et al., 2005; Varshavsky, 1996). This process is catalyzed by a family of highly conserved enzymes known as methionine aminopeptidases (MetAPs). There are two types of MetAPs: type 1 (MetAP1) and type 2 (MetAP2), which differ by the presence of a unique insertion of about 60 amino acids toward the C terminus of the catalytic domain in MetAP2. The prototypical MetAP1 and MetAP2 from prokaryotic bacteria and archaea contain the minimal catalytic domain. In contrast, eukaryotic MetAPs possess additional N-terminal domains. Thus, eukaryotic MetAP1 contains an N-terminal zinc finger motif that was shown to be involved in association with the ribosome in yeast (Vetro and Chang, 2002). Eukaryotic MetAP2 possesses an N-terminal poly-basic and acidic repeats that were shown to inhibit phosphorylation of eukaryotic initiation factor 2α subunit and enhance the rate of global protein synthesis (Datta et al., 1989, 2001, 2003a, 2003b, 2006, 2007; Ghosh et al., 2006). Several lines of evidence suggest that MetAP1 is constitutively expressed and is more likely to be a housekeeping enzyme, whereas MetAP2 expression is subject to regulation by cell cycle status and other extracellular signals (Chatterjee et al., 1998; Gupta et al., 1997). The importance of this family of enzymes is underscored by the lethal phenotypes of knockouts in both bacteria and yeast (Chang et al., 1989; Li and Chang, 1995).

Given their critical roles in cell viability in bacteria and lower eukaryotes, MetAPs have become attractive targets for developing drugs against bacteria and other pathological organisms. Considerable efforts have been made by many laboratories including ours to search for selective MetAP inhibitors for various pathologic organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Encephalitozoon intestinalis*, and *Plasmodium falciparum* (Chen et al., 2006; Didier, 1997; Huang et al., 2007; Oefner et al., 2003). In multicellular eukaryotic organisms, different isoforms of MetAP enzymes appear to have diverged in their functions and evolved tissue- and cell type-specific functions. For example, MetAP2 was identified as a relevant target for the fumagillin family of antiangiogenic natural products and was subsequently shown to play an important role in endothelial cell proliferation and angiogenesis (Griffith et al., 1997; Sin et al., 1997). More recently, human MetAP1 was shown to be involved in the control of cell cycle progression in the G2/M phase (Hu et al., 2006).

Malaria causes 1 to 2 million deaths each year in the developing world, with most mortality occurring among children. The emergence and rapid spread of drug resistance to existing antimalarial drugs such as chloroquine has posed a significant challenge to controlling the disease. There is an urgent need to develop new

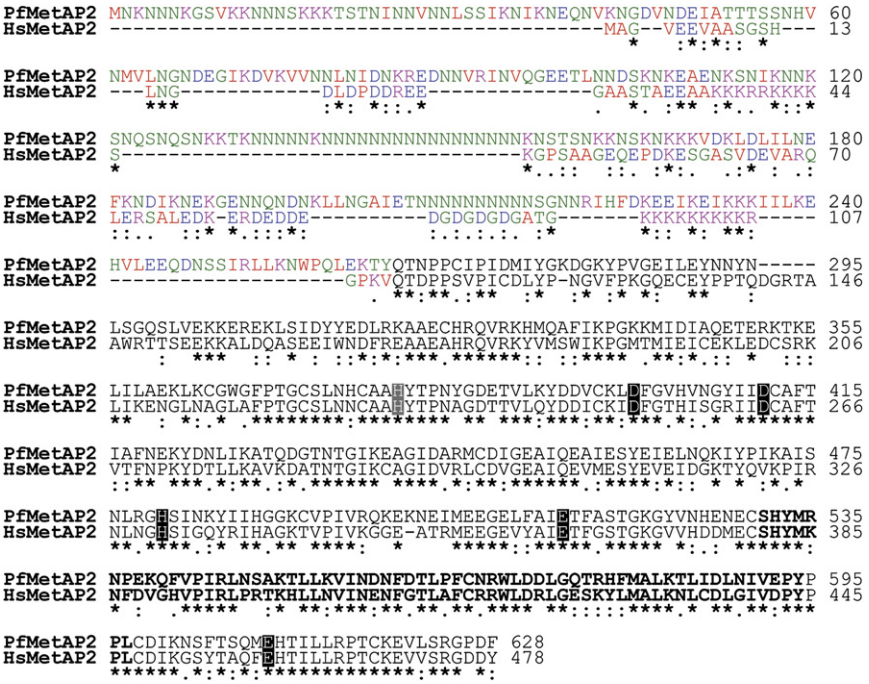


Figure 1. The Deduced PfMetAP2 Protein Sequence Shares High Identity to HsMetAP2

Protein sequence alignment using the Clustalw2 program (www.ebi.ac.uk/Tools). Red (AVFPMILW) indicates small amino acids (small + hydrophobic [including aromatic −Y]), blue (DE) indicates acidic, magenta (RK) indicates basic, and green (STYHCNGQ) indicates hydroxyl + amine + basic − Q. An asterisk (*) means that the residues in that column are identical, a colon (:) indicates conserved substitutions, and a period (.) indicates semiconserved substitutions. The human Histidine 231 (His231) and malaria His380 appear in white on a gray background. The five amino acids responsible for metal coordination appear in white on a black background.

antimalarial drugs, particularly those that can overcome resistance to existing drugs. Chiang and colleagues first reported that fumagillin and its synthetic analog TNP-470 are potent inhibitors of malaria parasite growth in cell culture, raising the possibility that fumagillin and analogs may be exploited as novel antimalarial agents (Zhang et al., 2002). Although malaria *P. falciparum* MetAP2 (PfMetAP2) was suggested as the likely target, there was no direct evidence in support of the proposition. In addition, a BLAST search of the *P. falciparum* (3D7) genome revealed an ortholog of HsMetAP2, PfMetAP2 (GeneDB accession number PF14_0327), whose protein sequence is longer than the one reported earlier (Zhang et al., 2002). As shown in Figure 1, there are 274 additional amino acids at the N terminus ($_{1}$MNK-CIPID$_{274}$), although the five amino acids (2 Asp, 1 His, and 2 Glu) responsible for metal coordination at the active site of all known MetAP enzymes are all conserved in PfMetAP2 (highlighted as white on a black background). These observations left open the question of whether the putative PfMetAP2 protein is a physiologically relevant target for fumagillin and TNP-470.

While TNP-470 reached Phased II clinical trials for cancer treatment, its dose-limiting neurotoxicity and extremely short half-life in vivo prevented its further advancement (Bhargava et al., 1999; Kruger and Figg, 2000). In attempts to reduce the toxicity and improve the pharmacological activities of the fumagillin-based MetAP2 inhibitors, we and others have synthesized and tested several analogs of fumagillin. Among the new fumagillin analogs reported to date, one stood out for its distinct structure and ability to reversibly associate with HsMetAP2 and lower toxicity in animals. It is known as fumarranol due to its rearranged core structure derived from the fumagillin backbone. In contrast to TNP-470 and the majority of known analogs of fumagillin, fumarranol is a noncovalent inhibitor of HsMetAP2 (Lu et al., 2006). Despite its reduced potency against HsMetAP2 (IC_{50} = 3 μM), it is quite active in an endothelial cell proliferation assay, with an IC_{50} of 34 nM (Lu et al., 2006). Significantly, a 10-day treatment with fumarranol at 90 mg/kg/day showed an antiangiogenesis potency comparable to TNP-470 at 30 mg/kg/day in a mouse angiogenesis model. Moreover, treatment of fumarranol did not cause the skin lesions that were seen in the TNP-470 group. The known antimalarial activity of fumagillin and TNP-470 prompted us to determine whether fumarranol also possesses antimalarial activity, and if so, whether it works through inhibition of PfMetAP2.

We herein disclose our findings on the expression, purification, and characterization of PfMetAP2 as well as its interaction with fumagillin and analogs. It was shown that recombinant PfMetAP2 from both *E. coli* and yeast are inactive in the MetAP enzymatic assay using an oligopeptide substrate in vitro. Using a biotin-fumagillin conjugate, however, we were able to pull down recombinant PfMetAP2 expressed in yeast, demonstrating their direct interaction. This interaction was further confirmed in a mammalian three-hybrid system in cells. We also found that fumarranol binds to PfMetAP2 and inhibits malaria growth in vitro. An SAR study with fumarranol analogs revealed a correlation between binding affinities for PfMetAP2 and inhibition of malaria growth in culture. Importantly, fumarranol was also found to inhibit growth of malaria parasite in vivo in a rodent malaria model.

RESULTS

The Deduced PfMetAP2 Protein Sequence Shared High Sequence Identity with HsMetAP2

The C-terminal catalytic domain of PfMetAP2 (265-628) and HsMetAP2 (112-478) share 58% sequence identity. The characteristic 64-amino acid insertion in HsMetAP2 (highlighted in bold and black) is highly conserved in PfMetAP2. HsMetAP2 contains one acidic domain ($_{77}$EDKERDEDDEDGDGDGDG$_{94}$, in blue) and two basic domains ($_{36}$KKKRRKKKK$_{44}$ and $_{98}$KKKKKKKKKR$_{107}$ in magenta) at its N-terminal extension (Figure 1). These three charged domains were also conserved in PfMetAP2. Unlike HsMetAP2, however, the N-terminal noncatalytic domain of

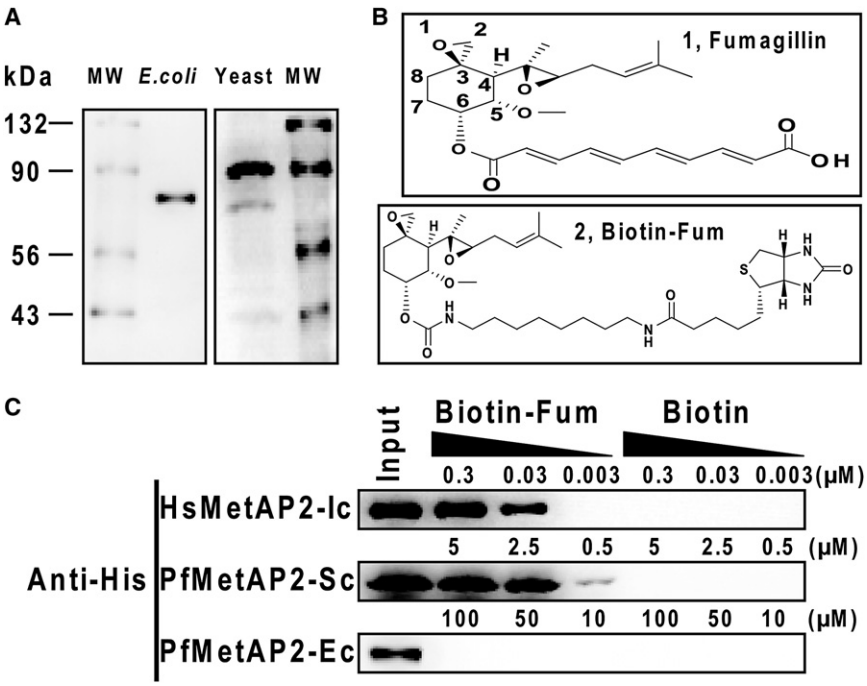


Figure 2. Biotin-Fum Binds to PfMetAP2-Sc but Not PfMetAP2-Ec

(A) Purified PfMetAP2-Ec (from *E. coli*) and PfMetAP2-Sc (from yeast) was detected on western blot analysis using anti-His-HRP antibody.

(B) Structures of fumagillin and biotin-Fum.

(C) Biotin-Fum but not biotin dose-dependently bound to HsMetAP2-Ic (from insect cell) and PfMetAP2-Sc with different affinity. Biotin-Fum up to 100 μM did not bind to PfMetAP2-Ec.

PfMetAP2 contains multiple stretches of polyasparagine, which are characteristic of malarial proteins. Differences in amino acids are also found throughout the catalytic domain between PfMetAP2 and HsMetAP2. In particular, residue K501 of PfMetAP2 was absent in HsMetAP2, likely leading to significant difference in the active sites between the two enzymes.

Biotin-Fum Binds Recombinant PfMetAP2 Overexpressed and Purified from Yeast but Not from *E. coli*

HsMetAP2, also known as p67, was reported to contain glycosylation sites (Datta et al., 2001, 2003a, 2007). The recombinant HsMetAP2 overexpressed and purified from *E. coli* lacked glycosylation with an apparent molecular weight of 53 kDa on SDS-PAGE and was found to be inactive. In contrast, recombinant HsMetAP2 overexpressed and purified from insect cells was active, with an apparent molecular weight of approximately 71 kDa (with a 4-kDa 6xHis tag) on SDS-PAGE (Griffith et al., 1998; Li and Chang, 1996). Similarly, PfMetAP2 overexpressed in *E. coli* had an apparent molecular weight of 72 kDa, whereas PfMetAP2 overexpressed in yeast was approximately 90 kDa (containing a 4-kDa Xpress epitope™ and 6xHis tag) (Figure 2A). To determine whether fumagillin (Figure 2B) can bind to recombinant PfMetAP2, a biotinylated fumagillin (biotin-Fum) (Figure 2B) was used in a pull-down assay with HsMetAP2 as a positive control (Griffith et al., 1997). Whereas biotin alone could not pull down either HsMetAP2 or PfMetAP2-Sc, biotin-Fum dose-dependently pulled down both HsMetAP2 and PfMetAP2-Sc. The relative binding affinity of biotin-Fum for PfMetAP2-Sc was approximately 50-fold weaker than that for HsMetAP2 judging from the minimal concentrations of biotin-Fum that were required to detect each enzyme (Figure 2C). In contrast, biotin-Fum failed to pull down PfMetAP2-Ec even at 100 μM.

The Binding of Biotin-Fum to Recombinant PfMetAP2-Sc Is Noncovalent

Fumagillin and TNP-470 have been shown to bind to HsMetAP2 covalently (Griffith et al., 1997; Sin et al., 1997). The covalent interaction between fumagillin or biotin-Fum conjugate and HsMetAP2 can withstand denaturation conditions in SDS-PAGE gel loading buffer in boiling water, enabling detection using a near-western blot assay (Griffith et al., 1997). To determine whether the binding of biotin-Fum to PfMetAP2 is also covalent, the PfMetAP2-Sc was incubated with biotin-Fum for 2 hr and the mixture was then heated in boiling water for 10 min before SDS-PAGE followed by near-western blot assay. As shown in Figure 3A, while the binding of biotin-Fum to the HsMetAP2 was covalent, the binding to PfMetAP2-Sc was not. No biotin signal could be detected from PfMetAP2-Sc samples with up to 100 μM biotin-Fum (Figure 3A). The reversible interaction between PfMetAP2 and fumagillin was also verified by examining the stability of the complex between biotin-fum and PfMetAP2 upon dialysis. The remaining complex between biotin-Fum and MetAP2 was captured with streptavidin beads and detected using antibodies against the 6XHis tag in a western blot. As shown in Figure 3B, while the complex between biotin-Fum and HsMetAP2 remained intact during dialysis, the PfMetAP2 complex with biotin-Fum could no longer be detected, consistent with the noncovalent and reversible binding of biotin-Fum to PfMetAP2.

Verification of the Interaction Between Fumagillin and PfMetAP2 Using the Mammalian Three-Hybrid System In Vivo

For reasons that remain unknown, PfMetAP2 expressed and purified from yeast or insect cells was not active in enzymatic assay (data not shown). To facilitate SAR studies, we decided to establish a mammalian three-hybrid system that is based on a yeast three-hybrid system (Licitra and Liu, 1996). The hybrid ligand in this system is a heterodimer between fumagillin (Fum) and dexamethasone (Dex). To ensure that the Dex-Fum heterodimer retained its ability to interact with PfMetAP2, one heterodimer, Fum-12O2-Dex4 (Figure 4A) was tested as a competitor in a biotin-Fum mediated pull-down assay using recombinant PfMetAP2 purified from yeast. PfMetAP2-Sc was thus incubated with various concentrations of Fum-12O2-Dex4 for 2 hr before biotin-Fum was added to capture free PfMetAP2. As shown in

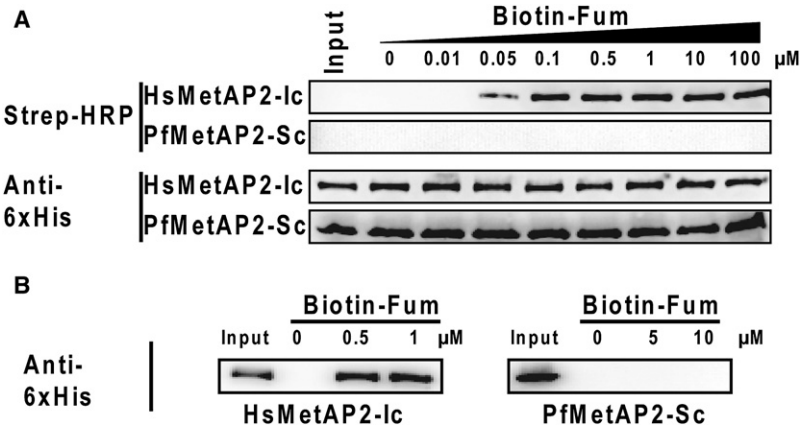


Figure 3. The Binding of Biotin-Fum Is Noncovalent to PfMetAP2-Sc

MetAP2 was incubated with different concentrations of biotin-Fum for 2 hr.

(A) SDS loading buffer was added to the mixture with 10 min of boiling. The sample was subjected to SDS-PAGE and detected for biotin signal with strep-hrp followed by anti-6xHis antibody to check the loading, or (B) the mixture was dialyzed against the binding buffer overnight at 4°C before the 2-hr capture by streptavidin sepharose beads. The MetAP2 on the beads was detected on western blot analysis using anti-His-HRP antibody.

Figure 4B, the preincubation with Fum-12O2-Dex4, but not Dex alone, competed for the binding of PfMetAP2 to biotin-Fum in a dose-dependent manner. This indicated that tethering of the linker and dexamethasone in Fum-12O2-Dex4 did not affect the ability of the fumagillin moiety of the heterodimer to interact with PfMetAP2 in vitro.

We made two constructs, pVP16-PfMetAP2 and pM-rGRHBD (524-796) (Chen et al., 2006), and cotransfected them into 293T cells. If Fum-12O2-Dex4 could bind to both VP16AD-PfMetAP2 and Gal4DBD-rGRHBD(524-796), the VP16AD (transactivation domain) and the Gal4DBD (DNA-binding domain) would be brought together, causing expression of Gal4-DNA binding site-driven luciferase reporter gene. Indeed, Fum-12O2-Dex4 was able to activate the expression of the luciferase reporter gene (Figure 5B). The biphasic bell-shaped dose-response curve revealed an optimal activating concentration of the Dex-Fum dimer at 0.3 μM. This is expected, because upon saturation of the two hybrid proteins, VP16AD-PfMetAP2 and Gal4DBD-rGRHBD, further increase in the concentration of Dex-Fum will lead to self-competition between the free Fum-Dex heterodimer and those present in the transcriptionally active VP16AD-PfMetAP2/Dex-Fum/Gal4DBD-rGRHBD ternary complex and dissociation of the ternary complex into transcriptionally inactive dimeric complex between Dex-Fum and either of the hybrid protein receptors. The ability of the Dex-Fum heterodimer to activate transcription of the reporter gene suggests that Dex-Fum binds to PfMetAP2 in vivo in mammalian cells, providing further support to the notion that fumagillin and analogs can directly bind to PfMetAP2.

Fumarranol Interacted with PfMetAP2 In Vivo

To determine whether fumarranol (Figure 5B) could bind to PfMetAP2, we performed the Dex-Fum-mediated mammalian three-hybrid assay in the absence and the presence of fumarranol. Thus, 24 hr after cotransfection with the three-hybrid plasmids, 293T cells were treated with different concentrations of potential competitors, including fumarranol for 4 hr, followed by a 48-hr incubation with 0.3 μM Fum-12O2-Dex4. As previously shown (Chen et al., 2006), TNP-470 inhibited the luciferase reporter gene activation (Figure 5C). Similarly, fumarranol also inhibited the induction of luciferase by the Dex-Fum heterodimer in a dose-dependent manner. The IC_{50} for fumarranol was estimated to be 116 μM in comparison to 2 μM for TNP-470 (Table 1). The ability of fumarranol to compete against Dex-Fum in this assay clearly suggests that, like fumagillin and Dex-Fum, it is also capable of binding to PfMetAP2. The roughly 58-fold lower potency of fumarranol than that of TNP-470 in this competition assay corroborates with its lower potency in parasite proliferation assay in comparison with TNP-470. In addition to fumarranol, we also determined the IC_{50} values of other fumarranol analogs in this competition assay. They are all above 500 μM, consistent with their lower potency against malaria parasites than fumarranol (Table 1).

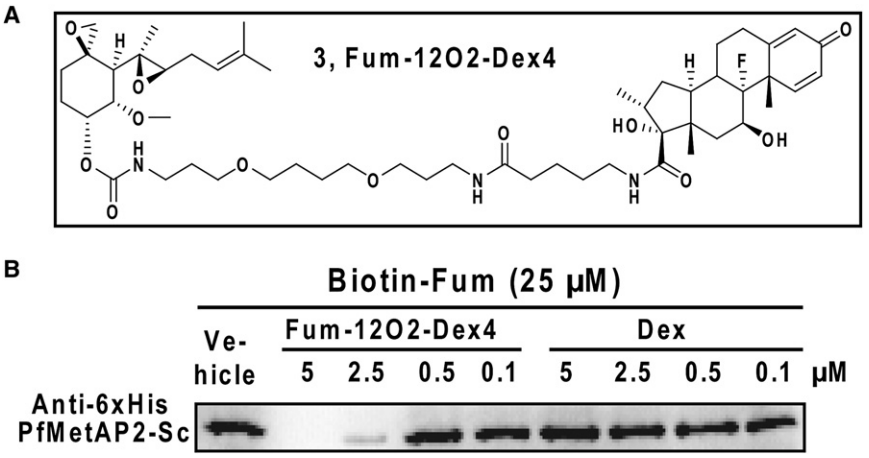


Figure 4. Fum-12O2-Dex4 but not Dex Dose-Dependently Competed the Binding of Biotin-Fum (25 mM) to PfMetAP2-Sc

(A) Structure of Fum-12O2-Dex4.

(B) PfMetAP2-Sc was incubated with different concentrations of Fum-12O2-Dex4 or Dex for 2 hr before addition of biotin-Fum to capture all the free PfMetAP2-Sc. All PfMetAP2-Sc bound to biotin-Fum were captured by streptavidin beads and detected by anti-6xHis antibody on western blot analysis.

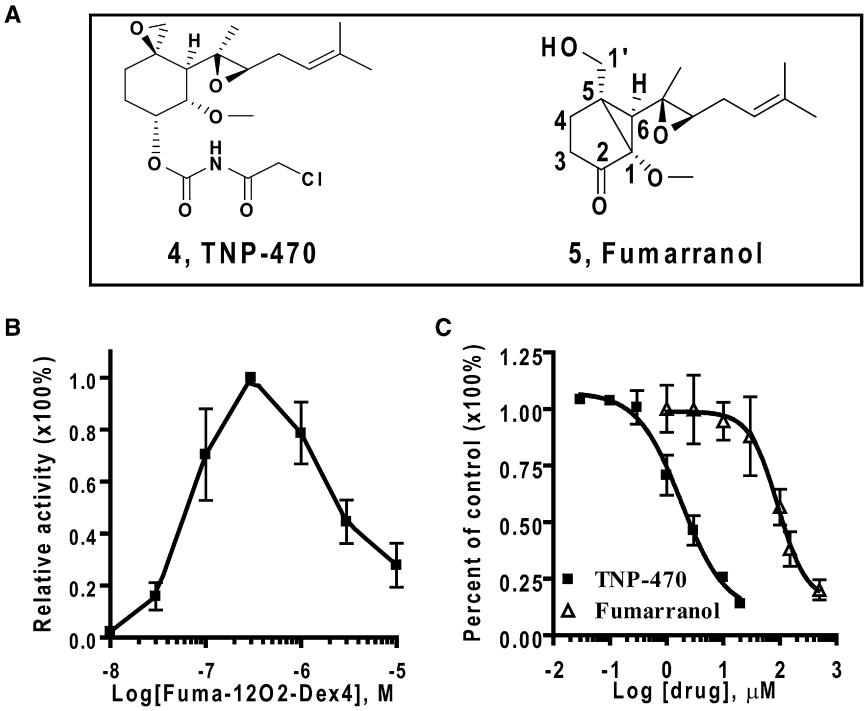


Figure 5. Fumarranol Could Inhibit Fum-12O2-Dex4-Activated Expression of Luciferase in a Mammalian Three-Hybrid System

(A) Structures of TNP-470 and fumarranol.

(B) Setup of the mammalian three-hybrid system. Dose response of Fum-12O2-Dex4 in inducting the activation of luciferase reporter gene in 293T cells cotransformed with pVP16-PfMetAP2 and pM-rGRHBD(524-796).

(C) Dose-dependent competition effect of TNP-470 and fumarranol on the activation of luciferase reporter gene by 0.3 μM Fum-12O2-Dex4. Data are expressed as the mean ± SD.

Fumarranol Inhibited Proliferation of both Drug-Sensitive and Drug-Resistant *P. falciparum* Parasites in Culture

We determined the effect of fumarranol on the growth of both NF54 (drug-sensitive) and W2 (multidrug-resistant) strains. Fumarranol was found to be active in blocking the proliferation of both strains with IC_{50} values of 0.16 and 0.27 μM, respectively (Table 1). In comparison with TNP-470, the potency of fumarranol is 80- to 100-fold lower. We also tested other fumarranol analogs in the malaria parasite proliferation assay. None of the analogs exhibited higher activity than fumarranol itself.

Fumarranol Cured Mice Infected with Chloroquine Resistant *P. yoelii* Parasites

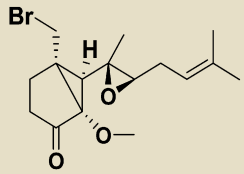
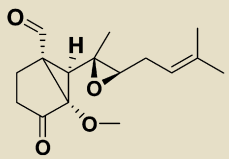
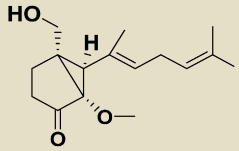
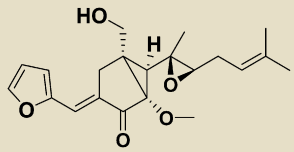
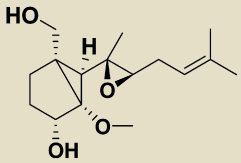
Fumarranol was further tested for its anti-malaria activity in vivo. Mice were given fumarranol once a day for 4 days starting 1 hour after infection with *P. yoelii* 17X lethal strain (chloroquine resistant). Fumarranol at 60 and 120 mg/kg significantly inhibited the parasitemia level on day 4 (Figure 6A). Fumarranol at 60 mg/kg had a potency comparable to TNP-470 at 20 mg/kg. As expected, chloroquine failed to completely eradicate the parasitemia at a dosage of 10 mg/kg, which indicated the existence of chloroquine resistance of the *P. yoelii* strain. After the 4-day drug treatment, mice were kept for another 26 days to determine the mean survival time. All surviving mice were checked for parasitemia again to determine whether the parasites were completely cleared. Only those mice without detectable parasitemia at day 30 after infection were considered cured. As shown in Figure 6B, fumarranol extended the mean survival time in a dose-dependent manner. At 120 mg/kg, fumarranol was more efficacious than either TNP-470 at 20 mg/kg or chloroquine at 10 mg/kg. Whereas only one mouse survived on day 30 in the 60 mg/kg fumarranol group and the 20 mg/kg TNP-470 group, 2 and 3 mice survived in the chloroquine (10 mg/kg) group and fumarranol (120 mg/kg) group, respectively. In addition, two mice from the 120 mg/kg fumarranol-treatment group were cured, whereas all other surviving mice still had parasitemia between 0.1% and 5% on day 30. Meanwhile, no skin lesion or abnormal behavioral patterns (e.g., fatigue, stress, or aggressiveness) were observed among the mice with fumarranol treatment, indicating that fumarranol is well tolerated by the mice.

DISCUSSION

In a previous study, an open reading frame was identified from the *P. falciparum* genome that showed high sequence identity with MetAP2 proteins from other eukaryotic organisms (Zhang et al., 2002), which was assigned as the putative malarial ortholog of human MetAP2. It was hypothesized that the antimalarial activity of fumagillin and TNP-470 is mediated by inhibition of the PfMetAP2 enzyme. One unusual feature of the previously identified PfMetAP2 protein is that it lacked the unique N-terminal extension of all other eukaryotic MetAP2 proteins, which contain the domains rich in acidic or basic amino acids (Zhang et al., 2002). It had appeared that malaria might have lost the unique N-terminal extension during evolution. A careful search in the *P. falciparum* database, however, revealed that the PfMetAP2 gene does encode an N-terminal extension beyond the core catalytic domain. Similar to other eukaryotic MetAP2 proteins, PfMetAP2 also contains stretches of poly-acidic and poly-basic residues. Unlike MetAP2 proteins from other organisms, PfMetAP2 contains poly-asparagine sequences within the N-terminal domain, characteristic of other malarial proteins (Barale et al., 1997; Bastien et al., 2004).

Although it has been shown previously that fumagillin and TNP-470 both possess potent inhibitory effects on malaria growth in vitro, and it has been speculated that PfMetAP2 is a likely target, no experimental evidence exists to support this hypothesis. In this study, we attempted to overexpress and purify PfMetAP2 in a heterologous expression system and assess the interaction between fumagillin and recombinant PfMetAP2. Similar to human MetAP2, PfMetAP2 expressed and purified

Table 1. Affinity Assay of Fumarranol Analogs with PfMetAP2 using Mammalian Three-Hybrid System and the Effect of Fumarranol Analogs on PfMetAP1a/b/c Enzymatic Activity and Proliferation of *P. falciparum* NF54 and W2 Strains In Vitro

		IC_{50} (μM)			
Compound		PfMetAP2	PfMetAP1a/b/c[a]	NF54	W2
4	TNP-470	2.0 ± 1.0	>300[a]	0.002 ± 0.0003	0.003 ± 0.0003
5	Fumarranol	116 ± 27	>300[a]	0.16 ± 0.03	0.27 ± 0.07
6		>500	ND	6.3 ± 1	8.8 ± 0.9
7		>500	ND	1.6 ± 0.5	1.6 ± 0.5
8		>500	ND	71 ± 7	>100
9		>500	ND	>100	>100
10		>500	ND	>100	>100

Data are expressed as the mean ± SD.
ND, not determined.
[a] Due to the limitation of solubility in the enzyme assay for PfMetAP1a/b/c, only up to 300 μM of the above compounds were tested.

from bacteria is inactive, perhaps due to lack of the appropriate posttranslational modifications in bacteria hosts (Baneyx and Mujacic, 2004). Whereas HsMetAP2 expressed in baculovirus-driven insect cells is fully active as an enzyme and binds fumagillin, PfMetAP2 expressed and purified from insect cells did not exhibit any enzymatic activity using a variety of N-terminal methionine-containing oligopeptide substrates (data not shown). It is possible that PfMetAP2 undergoes unique posttranslational modifications that cannot be recapitulated in insect cells or yeast. It is also possible that PfMetAP2 acts on a unique substrates within malarial parasites yet to be identified.

Despite the lack of enzymatic activity, the recombinant PfMetAP2 purified from either yeast or insect cells was able to bind to fumagillin as demonstrated by the pull-down assay using biotin-Fum. To further verify the interaction between fumagillin and PfMetAP2, we turned to a mammalian three-hybrid system that can be used to detect small molecule-protein interaction in mammalian cells. One advantage of the mammalian three-hybrid assay is that PfMetAP2 fusion protein is expressed in a mammalian host cells, allowing for the possible posttranslational modification. Another advantage of the mammalian three-hybrid system is that the signal in the form of activation

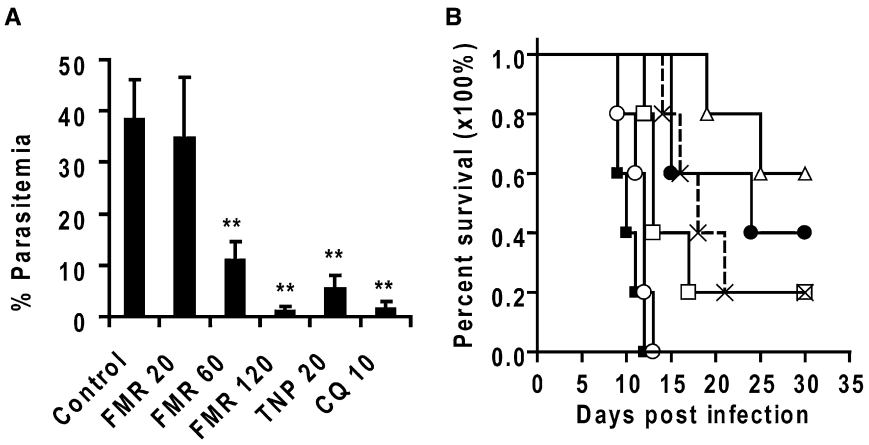


Figure 6. Antimalarial Activity of Fumarranol in Mouse Model

(A) Effect of subcutaneously administered fumarranol on parasitemia. C57BL/6 mice infected with *P. yoelii* 17 × lethal strain were given fumarranol (FMR 20, 60, or 120 mg/kg) or TNP-470 (TNP 20 mg/kg) or chloroquine (CQ 10 mg/kg) or equal volume of vehicle (control) once a day for 4 days. **$p < 0.01$ versus control.

(B) Effect of subcutaneously administered fumarranol on time of survival. ■ = control. ○ = FMR 20 mg/kg. □ = FMR 60 mg/kg. △ = FMR 120 mg/kg. × = TNP 20 mg/kg. ● = CQ 10 mg/kg. n = 5 in all groups. Data are expressed as the mean ± SD.

of a reporter gene is solely dependent on the interaction between PfMetAP2 and the fumagillin portion of the three-hybrid ligand, Dex-Fum conjugate. That the Dex-Fum conjugate is active both in competing against biotin-Fum for recombinant PfMetAP2 protein in vitro and activating the three-hybrid reporter gene in cells due in part to the direct binding of Dex-Fum to PfMetAP2 fusion protein provides strong support for the notion that fumagillin is capable of binding to PfMetAP2. TNP-470 and fumarranol were able to compete against Dex-Fum to block the reporter gene activation with potencies in line with their relative activity against malaria growth in vitro. The same was seen with a number of less active fumarranol analogs. The correlation between the ability to compete for PfMetAP2 binding in the mammalian three-hybrid system and to inhibit malaria growth in vitro is consistent with PfMetAP2 as a potential target for fumagillin, fumarranol, and their analogs. Additional genetic evidence, including genetic knockout of PfMetAP2, will be required to fully validate PfMetAP2 as the target for fumagillin and fumarranol.

Fumagillin and analogs were known to bind to human and yeast MetAP2 covalently through the modification of His231 in the active site of the enzyme (Griffith et al., 1998; Liu et al., 1998). This can be detected using the near-Western blot analysis with the help of biotin-Fum (Griffith et al., 1997). In contrast to HsMetAP2, the recombinant PfMetAP2 expressed in insect cells or yeast appeared to bind to fumagillin reversibly (Figure 3). It is noteworthy that the same His residue modified by fumagillin in human MetAP2 is conserved in PfMetAP2 (His380). Thus, the inability of fumagillin to covalently modify His380 in PfMetAP2 may likely be attributed to other residues in the active site of PfMetAP2 that differ from their counterpart of HsMetAP2. In addition, the insertion of a single residue, K501, in the catalytic domain of PfMetAP2, which is absent in HsMetAP2, could also render the structure of the active site of PfMetAP2 significantly different from that of HsMetAP2. It is thus likely that fumagillin is accommodated in the active site of PfMetAP2 in such a way that the spiro epoxide group can no longer reach the imidazole side chain of His380, as it does with HsMetAP2.

There are four different MetAP isoforms in *P. falciparum*: PfMetAP1a, PfMetAP1b, PfMetAP1c, and PfMetAP2. PfMetAP1a, PfMetAP1b, and PfMetAP1c all belong to the type 1 MetAP family. PfMetAP1b has an N-terminal domain bearing two zinc finger motifs that are absent from PfMetAP1a and PfMetAP1c. Whereas PfMetAP1a contains a minimal catalytic domain, PfMetAP1c has a long N-terminal domain that was predicted to contain a signal peptide for targeting PfMetAP1c into the apicoplast. All three type 1 PfMetAPs have been overexpressed and purified from *E. coli* and have been found to be enzymatically active (Chen et al., 2006). We thus tested TNP-470 and fumarranol against each of the three type 1 PfMetAPs using the coupled MetAP enzymatic assay (Chen et al., 2006). At a concentration of 300 μM, neither TNP-470 nor fumarranol affected the activity of PfMetAP1a, PfMetAP1b, or PfMetAP1c (Table 1), suggesting that TNP-470 as well as fumarranol are specific inhibitors of PfMetAP2 and bolstering the notion that inhibition of PfMetAP2 is likely the cause of inhibition of malarial growth.

Similar to TNP-470, the rearranged fumagillin analog fumarranol was found to be effective against both wild-type (clone NF54) and multidrug-resistant (W2) *P. falciparum* strains with roughly equal potency (Table 1). In comparison with TNP-470, fumarranol was about 80 fold less potent. This is consistent with the lower binding affinity of fumarranol for PfMetAP2 observed in the mammalian three-hybrid assay. To compare their potency in vivo, fumarranol and TNP-470 were tested in mice infected with *Plasmodium yoelii* 17X lethal strain (chloroquine resistant). As expected, at the same dose of 20 mg/kg, fumarranol was less effective than TNP-470 (Figure 6). However, an increase in fumarranol dose to 120 mg/kg yielded a better outcome than TNP-470 at 20 mg/kg or chloroquine at 10 mg/kg. Furthermore, three out five mice survived after 120 mg/kg fumarranol treatment, two of which were completely cured (free of parasites). Given that fumarranol exhibited much lower toxicity than TNP-470, these data suggest that fumarranol or its more potent analogs may be promising leads for developing antimalarial agents.

SIGNIFICANCE

The human MetAP2 has been shown to be the target for the fumagillin family of antiangiogenic natural products. Recent evidence suggests that fumagillin and its analog TNP-470 also possess potent antimalarial activity in vitro, likely through the inhibition of the malarial ortholog of HsMetAP2, PfMetAP2. However, there has been no direct experimental evidence supporting this hypothesis. Moreover, TNP-470 failed in human clinical trials due to its undesirable

pharmacological profiles, including short serum half-life and dose-limiting neurotoxicity, necessitating the identification of new inhibitors of PfMetAP2 as a lead. In this manuscript, we provide direct experimental evidence for the interaction between TNP-470 and PfMetAP2 by obtaining recombinant PfMetAP2 from yeast and insect cells. We demonstrate that unlike HsMetAP2, PfMetAP2 is bound to fumagillin in a reversible manner despite the conservation of an active site histidine residue that is known to be covalently modified by fumagillin and TNP-470, underscoring the difference in the active sites between HsMetAP2 and PfMetAP2. We show for the first time that TNP-470 is active in blocking malaria growth in vivo. We also found that fumarranol, an analog of fumagillin, retained both isoform specificity and the ability to inhibit malaria growth in vitro and in vivo. Together, these findings suggest that PfMetAP2 is a viable new target and fumarranol is a promising lead for developing new antimalarial agents.

EXPERIMENTAL PROCEDURES

Materials

All chemical reagents used in organic synthesis were purchased from Aldrich (St. Louis, MO). Biotin and dexamethasone were from Sigma (St. Louis, MO). Syntheses of biotin-labeled fumagillin (compound 1), TNP-470 (compound 3), fumarranol, and its derivatives (compound 4 to 9) were performed as described previously (Griffith et al., 1997; Lu et al., 2006). The Fum-Dex dimmer was synthesized using a previously reported procedure (Licitra and Liu, 1996). Each compound was confirmed by ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry. The details are reported in the Supplemental Material.

The vector pYES2/NTc and the host yeast strain INVSc1 were from Invitrogen (Carlsbad, CA). The vector pET29a and the host *E. coli* BL21 were from Novagen (San Diego, CA). The vectors pM and pVP16 for the mammalian three-hybrid system were from Clontech (Mountain View, CA). Construction of plasmids pVP16-PfMetAP2 and pM-rGRHBD (524-796) encoding vp16AD-PfMetAP2 and Gal4DBD-rGRHBD(524-796), the wild-type hormone-binding domain of rat glucocorticoid receptor (524-796), has been described (Chen et al., 2006).

Overexpression and Purification of Recombinant PfMetAP2 from *E. coli* and Yeast

The DNA sequence of the first seven amino acids $_{1}$MNKNNNK$_{7}$ has high similarity to $_{130}$KTKNNNN$_{136}$, which prevented us from cloning the full-length PfMetAP2. To circumvent the problem, the first five amino acids were skipped. The gene encoding PfMetAP2 (Δ1-5) was amplified from genomic cDNA of *P. falciparum* 3D7 strain. One pair of primers was used for cDNA amplification (forward primer, 5′-CGCGGATCCAATAAAGGTAGTGTTA-3′; reverse primer, 5′-CGCGAATTCTTAAAAGTCGGGACCACGAG-3′). The PCR fragment was cloned into pET28a and pYES2/NTc (same pair of restriction sites BamHI and EcoRI). The DNA inserts in those two plasmids were confirmed by sequencing prior to transformation into *E. coli* (BL21) and yeast (INVSc1) according to the manufacturer's instructions. The overexpression of PfMetAP2(6-628) from *E. coli* (or yeast) was induced with 1 mM IPTG (or 2% galactose plus 1% rafanose) at 16°C for 3 days. The cells were suspended in PBS (pH 7.5) and passed through a French press three times. Because both recombinant proteins have 6xHis tag, they were further purified with cobalt-based TALON affinity resin (Clontech) according to the manufacturer's instructions. The identity of purified recombinant proteins was verified on western blot analysis using anti-His-HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Biotin-Fum Affinity Binding Assay In Vitro

Recombinant PfMetAP2 from *E. coli* or yeast or recombinant HsMetAP2 (about 100 ng) was added to binding buffer (20 mM Tris-HCl [pH 7.1], 100 mM KCl, 0.2% Triton X-l 00, 20 μg/ml of leupeptin, aprotinin and 1 mg/ml of phenylmethyl-sulfonyl fluoride). They were incubated for 2 hr with biotin or biotin-Fum or ethanol at 4°C. Following incubation, 20 μl streptavidin sepharose (Pierce Biotech, Rockford, IL) was added and the mixture was incubated at 4°C for another 2 hr. The beads were collected by centrifugation and washed twice with 500 μl lysis buffer for 5 min. An aliquot of 40 μl of 1× SDS loading buffer was added; the samples were then boiled for 10 min, loaded onto a 12% SDS-PAGE gel, and subjected to western blot analysis. Proteins were identified using anti-His-HRP antibody. For competition assay, the proteins were first incubated with Fum-12O2-Dex4 or ethanol for 2 hr at 4°C before 25 μM biotin-Fum was added for the rest of experiment as described above.

To determine whether the binding is covalent on near-western blot assay, as reported previously (Griffith et al., 1997), 40 μl of 1× SDS loading buffer was directly added to the proteins after the 2-hr incubation with biotin-Fum or ethanol at 4°C. After being boiled for 10 min, the samples were analyzed using western blotting first with strep-HRP (GE Healthcare, Piscataway, NJ) (to check the biotinylation) and then stripped, followed by blotting with anti-His-HRP antibody (to check equal loading). Alternatively, the proteins after the 2-hr incubation with biotin-Fum or ethanol were dialyzed against 2 l of binding buffer overnight at 4°C. The remaining biotin-Fum-MetAP2 complex was captured by streptavidin sepharose beads. The MetAP2 proteins captured on the beads were detected with western blotting using anti-His-HRP antibody.

Establishment of a Mammalian Three-Hybrid System with Fum-12O2-Dex4 and its Application in Fumarranol SAR Studies

The system is similar to the previously reported system (Chen et al., 2006), with some modifications. In brief, plasmid pVP16-PfMetAP2 and pM-rGRHBD(524-796) were cotransfected into 293T cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. To determine whether Fum-12O2-Dex4 could induce expression of the reporter gene luciferase, 24 hr after cotransfection, the cells were incubated in a 24-well plate with different concentrations of Fum-12O2-Dex4 for another 48 hr. To determine the luciferase activity, cells in each well were washed three times with PBS (pH 7.5), transferred to Eppendorf tubes, and lysed with report lysis buffer (25 mM Tris [pH 7.8], 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton X-100). The lysate was subject to luciferase assay using bettle luceferin (Promega, Madison, WI) in luciferase reaction buffer (20 mM Tricine [pH 7.8], 1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 uM coenzyme A, 470 μM luciferin, and 530 μM ATP.) as a substrate. Upon mixture, the instantaneously generated chemiluminescence signal was determined by 1450 MicroBeta plate reader (Wallac, Waltham, MA) using a preset program. The protein concentration of each lysate was also determined using the Bradford assay (Bio-Rad, Hercules, CA). To compare the relative luciferase activity of each sample, the readout of chemiluminescence signals were first divided by their corresponding protein concentrations and then subtracted by background (no Fum-12O2-Dex4 treatment). The net luciferase activity of each sample thus obtained was normalized against the control to obtain the relative luciferase activity. To determine the ability of different drugs to bind PfMetAP2, they were preincubated with the cotransfected cells for 4 hr before the addition of 0.3 μM Fum-12O2-Dex4.

Antiproliferation Assay

P. falciparum NF54 (wild-type) and W2 strain (multidrug-resistant) were maintained at 37°C in RPMI 1640 medium with 10% human serum at 3%–5% hematocrit in a reduced oxygen environment produced by the candle-jar technique (Trager and Jensen, 1976). Parasites were incubated with test compounds for 24 hr followed by the [^{3}H]-hypoxanthine (GE Healthcare, NJ) incorporation assay described previously (Chen et al., 2006). Percentage of control (no drug treatment) was used to determine IC_{50} values.

Animal Experiments

C57BL/6 mice (female, 4-6 wk old) were obtained from NCI and treated in accordance with Johns Hopkins Animal Care and Use Committee procedures. Mice were infected with *P. yoelli* 17X lethal strain by intraperitoneal injection of 4×10^{6} parasitized erythrocytes. Test compounds were administered subcutaneously starting 1 hr post infection once a day for 4 days. Control mice were given vehicle only. Chloroquine and TNP-470 was used as positive control. Blood was taken from the tail 24 hr after the last drug administration on day 4, and blood smears were prepared and stained with Giemsa. Parasitemia

was determined microscopically by counting four fields of approximately 200 erythrocytes per filed. Data were shown as mean percentage of parasitemia ± SD. Mice that were alive on day 30 with no detectable parasitemia on slide were considered cured.

Statistics

IC_{50} values for drug competition assay in three-hybrid system and cell proliferation assays were determined using four-parameter logarithmic analysis with GraphPad Prism4 and are presented as the mean ± SD for triplicate experiments. For animal tests, p values were determined using the two-tailed Student's t test.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00030-1/.

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REFERENCES

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. Nat. Biotechnol. *22*, 1399–1408.

Barale, J.C., Candelle, D., Attal-Bonnefoy, G., Dehoux, P., Bonnefoy, S., Ridley, R., Pereira da Silva, L., and Langsley, G. (1997). Plasmodium falciparum AARP1, a giant protein containing repeated motifs rich in asparagine and aspartate residues, is associated with the infected erythrocyte membrane. Infect. Immun. *65*, 3003–3010.

Bastien, O., Lespinats, S., Roy, S., Metayer, K., Fertil, B., Codani, J.J., and Marechal, E. (2004). Analysis of the compositional biases in *Plasmodium falciparum* genome and proteome using Arabidopsis thaliana as a reference. Gene *336*, 163–173.

Bhargava, P., Marshall, J.L., Rizvi, N., Dahut, W., Yoe, J., Figuera, M., Phipps, K., Ong, V.S., Kato, A., and Hawkins, M.J. (1999). A Phase I and pharmacokinetic study of TNP-470 administered weekly to patients with advanced cancer. Clin. Cancer Res. *5*, 1989–1995.

Boutin, J.A. (1997). Myristoylation. Cell. Signal. *9*, 15–35.

Chang, S.Y., McGary, E.C., and Chang, S. (1989). Methionine aminopeptidase gene of *Escherichia coli* is essential for cell growth. J. Bacteriol. *171*, 4071–4072.

Chatterjee, M., Chatterjee, N., Datta, R., Datta, B., and Gupta, N.K. (1998). Expression and activity of p67 are induced during heat shock. Biochem. Biophys. Res. Commun. *249*, 113–117.

Chen, X., Chong, C.R., Shi, L., Yoshimoto, T., Sullivan, D.J., Jr., and Liu, J.O. (2006). Inhibitors of *Plasmodium falciparum* methionine aminopeptidase 1b possess antimalarial activity. Proc. Natl. Acad. Sci. USA *103*, 14548–14553.

Datta, B., Ray, M.K., Chakrabarti, D., Wylie, D.E., and Gupta, N.K. (1989). Glycosylation of eukaryotic peptide chain initiation factor 2 (eIF-2)-associated 67-kDa polypeptide (p67) and its possible role in the inhibition of eIF-2 kinase-catalyzed phosphorylation of the eIF-2 alpha-subunit. J. Biol. Chem. *264*, 20620–20624.

Datta, R., Choudhury, P., Bhattacharya, M., Soto Leon, F., Zhou, Y., and Datta, B. (2001). Protection of translation initiation factor eIF2 phosphorylation correlates with eIF2-associated glycoprotein p67 levels and requires the lysine-rich domain I of p67. Biochimie *83*, 919–931.

Datta, R., Choudhury, P., Ghosh, A., and Datta, B. (2003a). A glycosylation site, 60SGTS63, of p67 is required for its ability to regulate the phosphorylation and activity of eukaryotic initiation factor 2alpha. Biochemistry *42*, 5453–5460.

Datta, R., Tammali, R., and Datta, B. (2003b). Negative regulation of the protection of eIF2alpha phosphorylation activity by a unique acidic domain present at the N-terminus of p67. Exp. Cell Res. *283*, 237–246.

Datta, B., Datta, R., Ghosh, A., and Majumdar, A. (2006). The binding between p67 and eukaryotic initiation factor 2 plays important roles in the protection of eIF2alpha from phosphorylation by kinases. Arch. Biochem. Biophys. *452*, 138–148.

Datta, B., Ghosh, A., Majumdar, A., and Datta, R. (2007). Autoproteolysis of rat p67 generates several peptide fragments: the N-terminal fragment, p26, is required for the protection of eIF2alpha from phosphorylation. Biochemistry *46*, 3465–3475.

Didier, E.S. (1997). Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication in vitro. Antimicrob. Agents Chemother. *41*, 1541–1546.

Ghosh, A., Datta, R., Majumdar, A., Bhattacharya, M., and Datta, B. (2006). The N-terminal lysine residue-rich domain II and the 340-430 amino acid segment of eukaryotic initiation factor 2-associated glycoprotein p67 are the binding sites for the gamma-subunit of eIF2. Exp. Cell Res. *312*, 3184–3203.

Griffith, E.C., Su, Z., Turk, B.E., Chen, S., Chang, Y.H., Wu, Z., Biemann, K., and Liu, J.O. (1997). Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem. Biol. *4*, 461–471.

Griffith, E.C., Su, Z., Niwayama, S., Ramsay, C.A., Chang, Y.H., and Liu, J.O. (1998). Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. Proc. Natl. Acad. Sci. USA *95*, 15183–15188.

Gupta, S., Bose, A., Chatterjee, N., Saha, D., Wu, S., and Gupta, N.K. (1997). p67 transcription regulates translation in serum-starved and mitogen-activated KRC-7 cells. J. Biol. Chem. *272*, 12699–12704.

Hu, X., Addlagatta, A., Lu, J., Matthews, B.W., and Liu, J.O. (2006). Elucidation of the function of type 1 human methionine aminopeptidase during cell cycle progression. Proc. Natl. Acad. Sci. USA *103*, 18148–18153.

Hu, X., Dang, Y., Tenney, K., Crews, P., Tsai, C.W., Sixt, K.M., Cole, P.A., and Liu, J.O. (2007). Regulation of c-Src nonreceptor tyrosine kinase activity by bengamide A through inhibition of methionine aminopeptidases. Chem. Biol. *14*, 764–774.

Huang, M., Xie, S.X., Ma, Z.Q., Huang, Q.Q., Nan, F.J., and Ye, Q.Z. (2007). Inhibition of monometalated methionine aminopeptidase: inhibitor discovery and crystallographic analysis. J. Med. Chem. *50*, 5735–5742.

Kruger, E.A., and Figg, W.D. (2000). TNP-470: an angiogenesis inhibitor in clinical development for cancer. Expert Opin. Investig. Drugs *9*, 1383–1396.

Li, X., and Chang, Y.H. (1995). Amino-terminal protein processing in Saccharomyces cerevisiae is an essential function that requires two distinct methionine aminopeptidases. Proc. Natl. Acad. Sci. USA *92*, 12357–12361.

Li, X., and Chang, Y.H. (1996). Evidence that the human homologue of a rat initiation factor-2 associated protein (p67) is a methionine aminopeptidase. Biochem. Biophys. Res. Commun. *227*, 152–159.

Licitra, E.J., and Liu, J.O. (1996). A three-hybrid system for detecting small ligand-protein receptor interactions. Proc. Natl. Acad. Sci. USA *93*, 12817–12821.

Liu, S., Widom, J., Kemp, C.W., Crews, C.M., and Clardy, J. (1998). Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science *282*, 1324–1327.

Lu, J., Chong, C.R., Hu, X., and Liu, J.O. (2006). Fumarranol, a rearranged fumagillin analogue that inhibits angiogenesis in vivo. J. Med. Chem. *49*, 5645–5648.

Oefner, C., Douangamath, A., D'Arcy, A., Hafeli, S., Mareque, D., Mac Sweeney, A., Padilla, J., Pierau, S., Schulz, H., Thormann, M., et al. (2003). The 1.15A crystal structure of the *Staphylococcus aureus* methionyl-aminopeptidase and complexes with triazole based inhibitors. J. Mol. Biol. *332*, 13–21.

Perrier, J., Durand, A., Giardina, T., and Puigserver, A. (2005). Catabolism of intracellular N-terminal acetylated proteins: involvement of acylpeptide hydrolase and acylase. Biochimie *87*, 673–685.

Sin, N., Meng, L., Wang, M.Q., Wen, J.J., Bornmann, W.G., and Crews, C.M. (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc. Natl. Acad. Sci. USA *94*, 6099–6103.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science *193*, 673–675.

Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. Proc. Natl. Acad. Sci. USA *93*, 12142–12149.

Vetro, J.A., and Chang, Y.H. (2002). Yeast methionine aminopeptidase type 1 is ribosome-associated and requires its N-terminal zinc finger domain for normal function in vivo. J. Cell. Biochem. *85*, 678–688.

Zhang, P., Nicholson, D.E., Bujnicki, J.M., Su, X., Brendle, J.J., Ferdig, M., Kyle, D.E., Milhous, W.K., and Chiang, P.K. (2002). Angiogenesis inhibitors specific for methionine aminopeptidase 2 as drugs for malaria and leishmaniasis. J. Biomed. Sci. *9*, 34–40.

